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(54) PROCESS FOR PRODUCING WATER-SOLUBLE POLYSACCHARIDES OR FOR ENHANCING THE SOLUBILITY IN WATER OF POLYSACCHARIDES

(71) We, AJINOMOTO CO., INC., a corporation organised under the law of Japan, of No. 6, 1-chome, Kyobashi, Chuo-ku, Tokyo, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:–

This invention relates to a process for producing water-soluble polysaccharides, or for enhancing the solubility in water of polysaccharides.

Natural polysaccharides are useful as raw materials in various industries, for example the food and feed manufacturing industries.

Recently, certain polysaccharides have been found to be effective as anti-tumor agents having only minor side-effects. Many natural polysaccharides are insoluble or only slightly soluble in water, and they can be extracted, but only in low yield, from natural organic substances under severe conditions, such as in an alkaline or acid medium and at high temperature. Moreover, the insolubility or low solubility of polysaccharides is inconvenient in the administration of the polysaccharide as an anti-tumor agent.

It is known that polysaccharides can be made water-soluble by carboxymethylation, phosphorylation or partial hydrolysis. However, these reactions often cause the polysaccharides to lose their physiological properties, for example anti-tumor activity.

We have now found that polysaccharides derived from natural organic substances dissolve readily or more readily when they are immersed in an aqueous solution of urea or of an analogous compound, and that the ready solubility in water of the resulting polysaccharides remains after the treatment.

The present invention accordingly provides a process for producing a water-soluble polysaccharide or for enhancing the solubility in water of an already water-soluble polysaccharide, which process comprises immersing a natural polysaccharide, a chemical derivative of a natural polysaccharide, or a plant or microorganism containing a natural polysaccharide, in an aqueous solution containing a compound having the following general formula:

$$R^1-NR^2-C=X \quad (I)$$

$$R^3-NR^4$$

wherein X is an oxygen or sulphur atom or an NH group, and each of R^1, R^2, R^3 and R^4, which can be the same or different, is a hydrogen atom or an alkyl radical containing from 1 to 4 carbon atoms; and recovering the resulting water-soluble polysaccharide from the aqueous solution.

The polysaccharides which can be used in the production of the water-soluble polysaccharides according to the process of the invention include, for example, a polysaccharide extracted from a bacterium, a yeast, a ligneous material and a *Graminaceae* grass, and a β-1, 3-glucan produced from any such polysaccharide by oxidation, reduction and hydrolysis. Examples of polysaccharides which can be used in the present invention are pachyman extracted from *Poria cocos* Wolf, lentinan extracted from *Lentinus edodes*, the polysaccharides extracted from the microbiological species *Fomes applanatus, Coriolus versicolor, Corolus hirsutus, Pholiota nameko, Flammulina velutipes* and *Trametes sanguinea*, holocellulose which is only slightly soluble in water, xylan, inulin, water-insoluble starch and pachymaran. Pachymaran is produced from pachyman by oxidation, reduction and then hydrolysis.

Urea of the analogous compound which can be used to prepare the water-soluble polysaccharide is the compound having the $\begin{matrix}>N\\>N\end{matrix}>C=$

group, and includes urea, iminourea (guanidine), thiourea and their derivatives in which one or both hydrogen atoms of one or both of the amino groups is/are substituted by an alkyl group containing up to 4 carbon atoms. The aqueous solution of urea or the analogous compound preferably has a concentration of more than 2 moles of urea or analogous compound per litre. The solubilization is effective at any pH value.

Preferably one part by weight of a natural polysaccharide or a chemical derivative thereof, or a plant or microorganism containing the same is immersed in from 20 to 100 parts by weight of the aqueous solution of urea or the analogous compound. The resultant suspension or solution is stirred, usually for more than one hour. The solubilization may proceed without heating, but it is promoted by heating at a temperature below 150°C, preferably in the range from 40 to 70°C. Insoluble substances can be removed by centrifuging or filtration, and a water-miscible organic solvent in a volume of 2 to 5 times the volume of the filtrate can be added to the supernatant or to the filtrate to precipitate water-soluble polysaccharide. The precipitate formed can be filtered off, washed with a small amount of chilled water and aqueous alcoholic solution, and dried to yield a water-soluble polysaccharide powder.

The "planar structure" (which is dependent on molecular weight, constitutive soccharide, made of glycoside bonding and made of branch bonding) of the water-soluble polysaccharide obtained by the process of the present invention is not different from that of the polysaccharide starting material but the "higher structure" (miscell) (which is the dimensional structure dependent on hydrogen bonds or Van der Waal's forces between two molecules) is changed from ordered to more random form, and most of the anti-tumor activity of the starting polysaccharide remains in the water-soluble or solubility-enhanced polysaccharide.

The amount of dissolved, *i.e.* water-soluble, polysaccharide can be colorimetrically determined by the Phenol-Sulphuric acid method.

The following examples illustrate different embodiments of the present invention, some of which have been referred to above.

Example 1

One gram of pachyman powder which had been extracted from a fungus of *Poria cocos* Wolf with aqueous alkaline solution, was introduced into 50 ml of a 6.0 molar aqueous urea solution, and the resulting suspension was stirred for 5 hours during which time the temperature was maintained at 80°C. Residual insoluble materials were removed by passing the suspension through a Millipore filter; 48 ml of the resulting filtrate was mixed with 140 ml of methanol. The word "Millipore" is a registered Trade Mark. The precipitate which formed was collected by centrifuging, washed with a small amount of water, and dried *in vacuo* to hield 720 mg of amorphous powder. The total amount of the powder obtained was entirely soluble in 50 ml of water. The temperature of the 50 ml of water used in this and other examples for determining solubility was room temperature.

When, for comparison, the same procedure as described above except for the replacement of the aqueous urea solution by water was performed, no precipitate was formed although a volume of methanol which was 10 times that of the filtrate was added.

Since the solubility of natural pachyman in water is only about 1.8 mg/dl at 120°C, the solubility of the water-soluble pachyman produced above was about 800 times greater than that of natural pachyman.

Example 2

One gram of pachyman powder was suspended in 50 ml of 4*M* aqueous urea solution and another gram of pachyman powder was suspended in 50 ml of 8*M* aqueous urea solution; the two suspensions were treated in the same manner as described in Example 1, and 640 mg and 601 mg of dry amorphous powder were obtained, respectively. Each powdery product was entirely soluble in 50 ml of water.

Example 3

One gram of pachyman powder was suspended in 50 ml of a 3*M* guanidine hydrochloride solution, and the resulting suspension was stirred at 70°C for 24 hours, at the end of which time the suspension had turned into an almost clear solution. The solution obtained was treated in the same manner as described in Example 1, and 120 mg of an amorphous dry powder were obtained. The powder was entirely soluble in 50 ml of water.

Example 4

One gram of lentinan powder (which had been extracted from commercial *Lentinus edodes*) was introduced into 50 ml of a 6*M* aqueous urea solution, and stirred at 60°C for 24 hours. Insoluble substances present were removed by centrifuging and the supernatant was dialyzed against tap water for 48 hours, followed by dialysis against distilled water for 24 hours. The dialysis separated off the urea whilst leaving the water-soluble polysaccharide in the non-dialyzable solution. The non-dialyzable solution was mixed with 3 times its volume of methanol. The precipitate which formed was collected by centrifuging, and 720 mg of dry amorphous powder were obtained. The powder was entirely soluble in 50 ml of water, whereas 1 g. of the untreated lentinan was not soluble in 50 ml of water.

Example 5

One gram of pachymaran powder was introduced into 50 ml of a 4*M* aqueous urea solution, and kept at 80°C for 24 hours. The resulting solution was treated in the same manner as described in Example 1, and 70 mg of an amorphous dry powder were obtained.

The powder was entirely soluble in 50 ml of water, whereas the solubility of the untreated pachymaran in water was only about 5 mg/50 ml of water.

Example 6

One gram of xylan powder was introduced into 50 ml of a 4*M* aqueous urea solution, and treated in the same manner as described in Example 1; 220 mg of dry amorphous powder were obtained. The powder was entirely soluble in 50 ml of water, whereas only 45 mg of the untreated xylan could be dissolved in 50 ml of water.

Example 7

Ten grams of the mycelium of *Coriolus versicolor* were homogenized in 500 ml of a 4*M* aqueous urea solution, and stirred at 80°C for 24 hours. Insoluble substances were removed by filtration, and the filtrate was concentrated to 60 ml and dialyzed in the same way as described in Example 4. The non-dialyzable solution was mixed with 240 ml of methanol, and the precipitate which formed was filtered off and dried to a 590 mg of a polysaccharide in the form of an amorphous powder. The powder was entirely soluble in 50 ml of water.

When, for comparison, 10 g. of the same mycelium was homogenized in 500 ml of water (without any urea contained therein) and treated in the same manner as described above, except that the volume of the concentrated filtrate was 70 ml and the volume of the methanol mixed was 350 ml, the yield of the dry amorphous powder was only 160 mg.

Example 8

2.3 Grams of the dry mycelium of *Poria cocos* were introduced into 200 ml of a 6*M* aqueous urea solution, and stirred at 70°C for 24 hours. Insoluble substances were removed by filtration, the filtrate was concentrated to 50 ml, and the concentrate was dialzed against tap water for 48 hours, followed by dialysis against distilled water for 24 hours. The non-dialyzable solution was mixed with 3 times its volume of methanol, and the precipitate which formed was filtered off, washed successively with methanol, diethyl ether and acetone, and then dried to hield 740 mg of an amorphous powder. The powder was a water-soluble polysaccharide mainly composed of glucose units.

When, for comparison, 2.3 g. of the same mycelium were introduced into 400 ml of water and boiler for 48 hours, the insoluble substances were removed by filtration, and the filtrate was mixed with 5 times its volume of methanol, no precipitate was formed.

When, for further comparison, the aqueous urea solution was replaced by 1% aqueous sodium hydroxide solution in the procedure described above, 701 mg of the polysaccharide powder were obtained, but the insolubility of the polysaccharide was only 1.8 mg/dl at 120°C.

Example 9

50 Grams of *Coriolus hirsutus* (produced at Shiraoi town, Hokkaido) were introduced into 1 litre of a 6*M* aqueous urea solution, and stirred at 70°C for 5 hours. The solution was treated with the aqueous urea solution in the same manner as employed in Example 8, and the non-dialyzable solution was freeze-dried to yield 8.3 g. of water-soluble polysaccharide powder.

If, for comparison, the aqueous urea solution was replaced by 4 litres of water in the procedure described above, only 5.9 g. of the polysaccharide powder were obtained.

Example 10

60 Grams of defatted *Poria cocos* were introduced into 2 litres of a 6*M* aqueous urea solution, and stirred at 100°C for 16 hours. Insoluble substances were removed by filtration, the filtrate was concentrated to 500 ml under reduced pressure, and the concentrate was mixed with 5 times its volume of methanol. The precipitate which formed was dialyzed in the same manner as employed in Example 8, and dried to a yield 23.3 g. of water-soluble pacyman powder.

Example 11

10 Grams of the mycelium of *Trametes sanguinea* were introduced into 500 ml of an 8*M* aqueous urea solution, ground and mixed with a homogenizer, and kept at 80°C for 20 hours with stirring. Insoluble substances were removed by filtration, 450 ml of the filtrate was concentrated to 70 ml, and the concentrate was mixed with 350 ml of methanol. The precipitate which formed was filtered off, washed with alcohol and dried to yield 1.7 g. of water-soluble polysaccharide having the β-1,3-glucoside linkage as the main chain.

When, for comparison, the aqueous urea solution was replaced by 500 ml of water, and the treatment was effected in the same manner as described above, the yield of the polysaccharide powder was only 160 mg.

Example 12

10 Grams of holocellulose (which is produced from plant tissue by elimination of fats and ligins) isolated from wheat straw were introduced into 500 ml of an 8*M* aqueous urea solution, and kept at 70°C for 24 hours with stirring. Insoluble substances were removed by filtration, the filtrate was dialyzed under the same conditions as described in example 4 and concentrated to 100 ml, and 400 ml of ethanol were added to the concentrated solution. The precipitate which formed was filtered off, washed with alcohol, and dried to yield 1.51 g. of water-soluble polysaccharide powder mainly composed of xylan.

When, for comparison, 10 g. of the same holocellulose were introduced into 1 litre of 1*N* sodium hydroxide solution instead of the aqueous urea solution, stirred at 25°C for 10 hours, and then treated in the same manner as described above, 11 g. of pllysaccharide powder were obtained. Only a small quantity of xylan was contained in the powder.

Example 13

20 Grams of crushed hemlock spruce defatted with acetone and dried previously were introduced into 2 litres of an 8*M* aqueous urea solution, and kept at 70°C for 24 hours with stirring. Insoluble substances were removed by filtration, the filtrate was concentrated to 100 ml under reduced pressure, the concentrate was dialyzed under the same conditions as described in example 4, and the dialyzed concentrate was mixed with 3 times its volume of ethanol. The precipitate which formed was filtered off, washed with diethyl ether, and dried to obtain 1.4 g. of water-soluble polysaccharide powder. The powder mainly contained galactoglucomanan.

When, for comparison, water was employed as the extracting agent instead of the aqueous urea solution, and the treatment was otherwise the same as described above, only 0.2 mg of dry galactoglucomannan powder with a low galactose content was obtained.

Example 14

500 Mg of pachyman were introduced into 50 ml of a 3*M* aqueous N-methylurea solution, and kept at 70°C for 24 hours with stirring. Insoluble substances were removed by filtration, and the filtrate was mixed with 50 ml of methanol. The precipitate which formed was collected, washed with methanol and diethyl ether, and then dried. The yield of the water-soluble pachyman was 408 mg.

Example 15

The procedure of Example 14 was repeated except that the aqueous N-methyl urea solution was replaced by a 3*M* aqueous solution of one of thiourea, N,N-dimethylurea, N,N'-dimethylurea, N,N,N',N'-tetramethylurea and 1,1,3,3-tetramethylguanidine hydrochloride. The yields of water-soluble pachyman were 442 mg, 224 mg, 170 mg, 329 mg and 102 mg, respectively, for the five different urea derivatives.

WHAT WE CLAIM IS:–

1. A process for producing a water-soluble polysaccharide or for enhancing the solubility in water of an already water-soluble polysaccharide, which process comprises immersing a natural polysaccharide, a chemical derivative of a natural polysaccharide, or a plant or micro-organism containing a natural polysaccharide, in an aqueous solution containing a compound having the following general formula:

$$R^1-NR^2-C=X \quad (I)$$
$$R^3-NR^4$$

wherein X is an oxygen or sulphur atom or an NH group, and each of R^1, R^2, R^3 and R^4, which can be the same or different, is a hydrogen atom or an alkyl radical containing from 1 to 4 carbon atoms; and recovering the resulting water-soluble polysaccharide from the aqueous solution.

2. A process according to Claim 1 wherein the polysaccharide starting material is a polysaccharide extracted from a bacterium, a yeast, a ligneous material or a *Graminaceae* grass.

3. A process according to Claim 2 wherein the polysaccharide starting material is pachyman, lentinan, a polysaccharide extracted from the microbiological species *Coriolus versicolor, Coriolus hirsutus* or *Trametes sanguinea*, a holocellulose, xylan or galactoglucomannan.

4. A process according to Claim 2 wherein the polysaccharide starting material is β-1,3-glucan.

5. A process according to any preceding claim wherein the concentration of the compound of formula I in the aqueous solution is more than 2 moles/litre.

6. A process according to any preceding claim wherein the polysaccharide starting material remains immersed for longer than one hour.

7. A process according to any preceding claim wherein the temperature of the solution is maintained in the range from 40 to 70°C.

8. A process according to any preceding claim wherein there is employed one part by weight of polysaccharide starting material per 20 to 100 parts by weight of the aqueous solution of the compound of formula I.

9. A process according to any preceding claim which includes, subsequent to the immersion, separating off insoluble substances from the aqueous solution, and then precipitating the resulting water-soluble polysaccharide from the aqueous solution by the addition of a water-miscible organic solvent.

10. A process according to Claim 1, substantially as described in any one of the foregoing Examples.

11. A water-soluble polysaccharide whenever produced by the process according to any preceding claim.

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